

COMPARATIVE STUDIES ON THE METABOLISM OF
l-CYSTINE AND *dl*-METHIONINE IN THE
ORGANISM OF THE RABBIT

BY ROBERT WALLACE VIRTUE

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIRE-
MENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE
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THE METABOLISM OF SULFUR

XXI. COMPARATIVE STUDIES OF THE METABOLISM OF *l*-CYSTINE AND *dl*-METHIONINE IN THE RABBIT*

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The extent of the oxidation of the sulfur of the amino acid, cystine, by the animal organism has been the subject of frequent study (1, 2). It has been shown that the sulfur of cystine administered in moderate quantities is oxidized readily and excreted in the urine chiefly as sulfate sulfur. Variable recoveries of the sulfur of cystine when fed are frequently obtained, owing presumably to the ready decomposition of cystine by the intestinal microflora with the formation of hydrogen sulfide and mercaptans. It has also been observed that, if the α -amino group of cystine is blocked by some chemical group which prevents ready deamination in the organism (*i.e.* acetyl, benzoyl, etc.), the oxidation of the sulfur of the molecule is prevented to a very considerable extent (2-5).

Our knowledge of the conditions for the oxidation of methionine, the second amino acid containing sulfur present in the protein molecule, is not extensive. Mueller (6), who first isolated methionine as a product of the hydrolysis of protein, observed in a limited series of experiments a ready oxidation of the sulfur of small quantities of methionine when fed to man. Pirie (7), after administering *l*-methionine equivalent to 320 mg. of sulfur to dogs, was able to recover 79 to 86 per cent of the sulfur fed as "extra" sulfur in the urine. A similar ready oxidation of the sulfur of *dl*-methionine by the dog has been noted by White and Lewis (8) and by Stekol and Schmidt (9).

* A preliminary report of this investigation was presented before the Twenty-seventh meeting of the American Society of Biological Chemists at Cincinnati, April 10-12, 1933 (*J. Biol. Chem.*, **100**, xev (1933)).

The object of the present investigation was to afford a comparison of the oxidation of the sulfur of *l*-cystine and *dl*-methionine in the organism of the rabbit. The effect of blocking the α -amino group of methionine by conjugation with the benzoyl group has also been studied.

EXPERIMENTAL

Male rabbits of 2 to 3 kilos weight were kept in the usual metabolism cages and fed uniformly 70 gm. of oats and 125 gm. of cabbage daily. This diet was eaten readily except on the days when the sulfur derivatives were administered, when part or, in some cases, all of the oats were occasionally not eaten. The urine was collected at regular intervals by gentle pressure on the abdominal wall. In some experiments, the samples of urine were collected in two portions (usually 6 and 18 hours) on the experimental days.

Total nitrogen of the urine was determined by the Kjeldahl-Gunning method, creatinine by Folin's micromethod, and the partition of urinary sulfur by the usual gravimetric procedures, the oxidizing reagent of Denis being employed in the determination of total sulfur. Disulfide linkages ($-\text{SS}-$) were determined by a modification (10) of the iodometric method of Okuda and the results were calculated as cystine. Urines were examined for protein and thiosulfates by the usual qualitative tests.

The determination of the specific rotation of the cystine, prepared from hair in this laboratory, gave a value, $[\alpha]_D^{20}$, of -219.4° . The methionine was a synthetic product. The *dl*-benzoylmethionine,¹ prepared according to the usual procedure for benzoylating amino acids, after purification melted at 150° , a melting point slightly higher than that reported for this compound by Windus and Marvel (11). α -Phenylureidocystine was prepared by the method of Patten (12). All compounds administered showed satisfactory values for sulfur and nitrogen on analysis.

The sodium salts of the various compounds were usually administered. In the earlier experiments the hydrochlorides of cystine and methionine were used. However, the substituted derivatives of methionine and cystine, the behavior of which it was desired to investigate, do not form easily soluble salts with hydrochloric acid,

¹ We are indebted to Dr. Julius White, National Research Council Fellow in Medicine, for the preparation of this compound.

while the alkali salts of these compounds are sufficiently soluble to permit their use readily. Therefore in order to insure a uniform procedure, it was decided to administer the sodium salts of the various amino acids and their derivatives. Oral administration was accomplished by the use of a small rubber catheter introduced into the stomach and intravenous injections were made into the marginal ear vein. If more than one substance were administered to the same animal, intervals of at least 4 days between experiments were allowed in order to obtain satisfactory normal control values.

DISCUSSION

A summary of all the experimental data concerned with the elimination of "extra" urinary sulfur after the administration of the various compounds under investigation is presented in Table I. The results obtained with cystine are similar to the results recorded in the literature (1, 2) and indicate a variable recovery of the sulfur of the cystine administered as extra urinary sulfur, the greater part of the "extra" sulfur appearing as "extra" sulfate sulfur, *i.e.* in oxidized form, except after intravenous injection. When the α -amino group of cystine was blocked by a group which prevented ready deamination as in α -phenylureidocystine, little evidence of the oxidation of the sulfur was obtained, most of the extra urinary sulfur being excreted as organic sulfur, a finding in confirmation of the earlier work of one of us (3).

The amount of extra sulfur appearing in the urine after methionine was fed or injected was of approximately the same order of magnitude as after cystine, with one exception. In one experiment, in which methionine hydrochloride was fed, only 28 per cent of the sulfur of the compound was excreted in the urine. Apart from the probability of a very active destruction of the material fed by the intestinal microflora, no explanation for the low recovery of the sulfur in this experiment can be offered. The sulfur of the methionine molecule appeared to be as readily oxidized as the sulfur of the cystine molecule, when administered in equivalent amounts. Even when the methionine was introduced parenterally, a ready oxidation was observed. In one experiment only, in which methionine hydrochloride was injected intravenously, was the oxidation of the sulfur poor. In this experiment (Table I), 57

TABLE I

Distribution of "Extra" Sulfur Excreted in Urine by the Rabbit after Administration of l-Cystine, dl-Methionine, and Their Derivatives

In all the experiments, the amounts of the various compounds used were such that the equivalent of 400 mg. of sulfur was administered. All the sulfur excretions are expressed as percentages of the total sulfur administered.

Compound	Mode of administration	Extra sulfur excreted		
		Total	Total sulfate	Organic
		per cent	per cent	per cent
Cystine hydrochloride	Oral	78	49	29
" "	"	62	25	37
" (sodium salt)	"	75	53	22
" " "	"	87	78	9
" " "	"	72	58	14
" " "	"	57	49	8
" " "	Intravenous	54	17	37
" " "	Subcutaneous	94	80	14
α -Phenylureidocystine (sodium salt) . . .	Oral	61	11	50
" " " "	"	54	10	44
" " " "	Subcutaneous	61	-7	68
" " " "	"	59	0	59
" " " "	"	68	0	68
Methionine hydrochloride	Oral	85	57	28
" " "	"	81	63	18
" " "	"	59	35	24
" " "	"	28	6	22
" " "	Intravenous	70	46	24
" " "	"	82	61	21
" " "	"	66	38	28
" " "	"	82	35	47
" (sodium salt)	Oral	61	32	29
" " "	"	89	64	25
" " "	Subcutaneous	67	40	27
" " "	"	54	54	0
" " "	"	67	51	16
" " "	"	82	72	10
α -Benzoylmethionine (sodium salt) . . .	"	59	2	57
" " " "	"	30	-3	33
" " " "	Intravenous	55	11	44

per cent of the extra sulfur appeared in the urine as organic sulfur (82 per cent of the sulfur of the compound fed was excreted, 35 per

cent as inorganic sulfate sulfur and 47 as organic sulfur). This ready oxidation of the sulfur of methionine is comparable to that observed by Pirie in the dog (7).

The most interesting observation made in the series of experiments in which methionine was administered was the appearance uniformly in the urine of the experimental days of a substance which gave a positive cyanide-nitroprusside test. This test, which is commonly described as a test for cystine, is given by any substance which on reduction yields a sulfhydryl group. However, when the Sullivan reaction, a reaction more specific for the presence of cystine, was applied to these urines, negative results were always obtained. This is believed to indicate the excretion of some compound containing the disulfide grouping other than cystine.

Further evidence of the presence of such a compound was obtained from the quantitative data. The disulfide groups, as determined by a modified iodometric method showed a slight but unmistakable rise on the days when methionine was fed or injected. A typical experiment is presented in Table II. On the 5th day, the disulfide calculated as cystine which had ranged from 10 to 21 mg. on the preliminary control days increased to 43 mg. following the subcutaneous injection of methionine. Similarly on the 19th day, the excretion of disulfides calculated as cystine was 84 mg. following the oral administration of methionine. Similar evidence of increased excretion of disulfide compounds was obtained in every experiment with methionine. Slight increases in the excretion of disulfide were observed also after the administration of cystine (*cf.* the 24th day, Table II). That the increased values in these experiments were due to the presence of cystine which had escaped oxidation was shown by weakly positive Sullivan tests.

On heating with sulfuric acid, methionine has been found to yield a product which gives a positive cyanide-nitroprusside test, but no Sullivan test for cystine. From the products of the reaction, Butz and du Vigneaud (13) were able to isolate and identify the next higher homologue of cystine, to which the name, homocystine, was given. In our experiments, the reactions of the urine after the administration of methionine can be explained on the basis of the excretion of small amounts of homocystine, as a product of the intermediary metabolism of methionine. We have

prepared homocystine from methionine, according to the directions of Butz and du Vigneaud and have shown that, as anticipated, homocystine behaves similarly to cystine in the iodometric titration, that the disulfide group may be reduced, and the mercapto group thus formed may be determined by this method. De-

TABLE II

Distribution of Urinary Sulfur after Administration of Methionine, Benzoylmethionine, and Cystine

Rabbit J, weight 2.5 kilos. All the compounds were administered as the sodium salts in equivalent amounts (equivalent to 400 mg. of sulfur).

Day	Total N	Total S	Total sulfate S	Organic S	S-S linkages calculated as cystine	Day	Total N	Total S	Total sulfate S	Organic S	S-S linkages calculated as cystine
	mg.	mg.	mg.	mg.	mg.		mg.	mg.	mg.	mg.	mg.
1	1295	219	159	60	16	15	1180	224	150	74	23
2	1043	178	131	47	10	16	1131	236	154	82	41
3	1304	211	156	55	21	17	1292	210	159	51	10
4	1285	186	112	74	10	18	1460	209	161	48	21
5	1292	333*	277	56	43	19	1512	556†	401	155	84
6	1246	288	236	52	24	20	1091	177	120	57	15
7	1204	197	151	46	15	21	1343	235	177	58	8
8	1239	145	95	50	18	22	1414	237	172	65	39
9	1448	161	125	36	13	23	1551	186	138	48	10
10	1565	143	115	28	7	24	2045	492§	405	87	37
11	1352	238	177	61	24	25	1635	287	211	76	20
12	1204	212	163	49	27	26	1570	188	143	45	16
13	1075	201	152	49	15	27	1948	270	204	66	16
14	1321	379†	146	233	24	28	1658	162	126	36	10

* 1.847 gm. of methionine were injected subcutaneously.

† 3.163 gm. of benzoylmethionine were injected subcutaneously.

‡ 1.847 gm. of methionine were given orally.

§ 1.5 gm. of cystine were injected subcutaneously.

methylation is not an uncommon reaction in the animal organism. We suggest tentatively that a primary reaction in the metabolism of methionine is demethylation to yield the next higher homologue of cysteine (homocysteine) and that 2 molecules of this compound by oxidation form homocystine which is excreted in small amounts in the urine. Confirmation of these experiments with rabbits was

obtained from studies with young white rats fed methionine by stomach tube (14). The urines of these animals gave positive cyanide-nitroprusside tests after the methionine administration.

It will be observed in Tables I and II, that normal oxidation of the sulfur of the methionine molecule in the animal organism did not occur when 1 hydrogen atom of the α -amino group was replaced by a benzoyl group. The extra sulfur excreted was practically all contained in the organic sulfur fraction and there was no rise in the oxidized or sulfate sulfur fraction comparable to that observed after the administration of methionine. Further evidence that the increased excretion of organic sulfur was due to the presence of α -benzoylmethionine in the urine was obtained by isolation of the compound from the urine secreted during the 6 hour period immediately following its administration. The material isolated was recrystallized and identified by its crystalline form and melting point. No depression of the melting point was observed when the recrystallized benzoylmethionine obtained from the urine was mixed with the pure substance prepared in the laboratory.

It is believed that oxidation of the sulfur of methionine, as of cystine, is related to the process of deamination of the amino acid and that if the α -amino group is blocked, normal oxidation of the sulfur is impossible. No cyanide-nitroprusside reaction could be obtained from the urines excreted after the administration of α -benzoylmethionine. If, as we believe, this reaction indicates the presence of homocystine in the urine as a product of the intermediary metabolism of methionine, it would appear that blocking the α -amino group of methionine not only results in failure of the normal oxidation of the sulfur, but that demethylation, which we have suggested as the initial stage in the metabolism of methionine, may also be prevented.

Du Vigneaud, Dyer, and Harmon (15) have recently shown that the growth of young white rats on a cystine-deficient diet may be increased by the addition of homocystine to the diet, thus furnishing additional evidence in support of a physiological relationship between methionine and homocystine. Their results are in harmony with the suggestion that demethylation with the formation of homocystine may occur in the intermediary metabolism of methionine.

As previously stated, creatinine determinations were carried out in all the experiments. No changes beyond the limit of experimental error of the method for creatinine determination were observed. This finding is not in accord with the observations of Beard and Barnes (16), who observed an increased excretion of creatinine of 28 per cent after the feeding of 1.5 gm. of cystine to white rats. It is to be noted however that in our experiments the amount fed per kilo of body weight was much less than in the studies of Beard and Barnes. Stekol and Schmidt (9) have reported an increased excretion of creatinine after feeding 1 to 3 gm. of methionine to dogs of approximately 13 kilos in weight. It is difficult to interpret these findings. In view of the fact that many substances other than creatinine react with picric acid in the presence of sodium hydroxide to give colored complexes, it is suggested that the reaction may have been due to some product of metabolism other than creatinine.

SUMMARY

1. *dl*-Methionine and *l*-cystine administered in equivalent amounts orally and subcutaneously (equivalent to 400 mg. of sulfur) to rabbits were catabolized readily, the major portion of the extra sulfur appearing in the urine as oxidized (sulfate) sulfur.

2. After the administration of *dl*-methionine to rabbits, a substance having the reactions of a disulfide was excreted in the urine. This substance gave a positive cyanide-nitroprusside reaction but no test for cystine (Sullivan).

3. When the amino group of methionine was blocked as in α -benzoylmethionine, oxidation by the organism of the rabbit was checked, the sulfur of the compound administered being excreted in the organic sulfur fraction of the urine. It appears that as with cystine (3-5), normal oxidation of the sulfur of methionine by the animal organism can occur only if the α -amino group is free. No excretion of a compound which gave a positive cyanide-nitroprusside test was observed after the parenteral administration of α -benzoylmethionine.

4. It is suggested that a primary reaction in the catabolism of methionine is demethylation and that the positive cyanide-nitroprusside tests obtained in the urine may be due to the presence of

small amounts of homocystine, formed by demethylation of methionine and oxidation of the resulting mercapto derivative to the disulfide.

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